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The hypothesis being tested is that the thioredoxin redox signaling system is essential for the growth of some human breast cancers and that drugs inhibiting this system will block oncogenesis and cause selective growth inhibition and/or apoptosis. The specific objectives of the IDEA proposal are to generate and identify selective inhibitors of thioredoxin using target-array chemistry methodologies, *in vitro* assays and cell-based screening approaches. The scope of the research activity demanded that we develop semi-automated synthetic methodology. We ultimately intend to select one or more lead compounds that could be optimized as candidates for clinical development, which would encompass a Clinical Translational Research (CTR) proposal. We will perform preclinical pharmacokinetics and formulation studies on any interesting candidates.

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Introduction

The hypothesis of this project continues to be that thioredoxin redox signaling systema are essential for the growth of some human breast cancers and that drugs inhibiting these systems will block oncogenesis and cause selective growth inhibition and/or apoptosis. The specific objectives of the IDEA proposal are to generate and identify selective inhibitors of thioredoxin using target-array chemistry methodologies, *in vitro* assays and cell-based screening approaches. The scope of the research activity demanded that we develop semi-automated synthetic methodology. We ultimately intend to select one or more lead compounds that could be optimized as candidates for clinical development, which would encompass a Clinical Translational Research (CTR) proposal. This report includes some previously reported as well as new information.

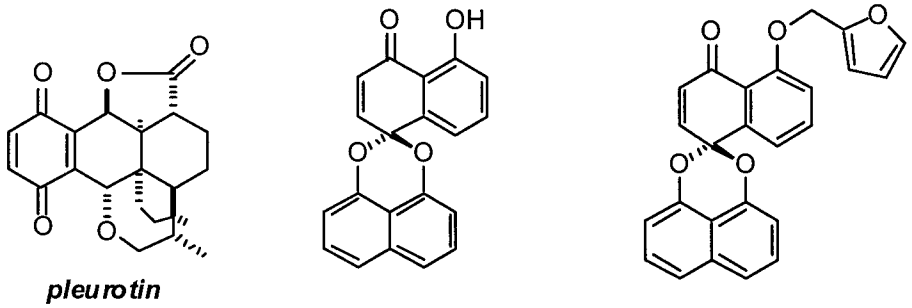
Body

Technical Objective #1. Synthesize libraries of small molecule compounds designed on the spiroketal naphthodecalin structure.

As mentioned in our previous report, we completed one of the main components of our first Technical Objective; namely to synthesize an initial library of approximately 50 compounds with maximal diversity in 5-20 mg quantities and to evaluate the purity and confirm the chemical structures of the library members. A peer review manuscript has been published on the spiroketal analogs [1].

During the last year we have made substantial progress toward the next goals of this grant. Our scientific goals were (1) the synthesis of 200-300 mg quantities of the submicromolar thioredoxin inhibitor palmarumycin CP_1 (Figure 1) as well as the novel lead structure SR-7 for biological evaluation and *in vivo* efficacy studies, and, (2) the total synthesis of the most potent thioredoxin inhibitor known to date, the natural product pleurotin. The latter project will form the basis for SAR studies of pleurotin analogues, and, hopefully, the identification of more selective, biologically active derivatives.

Figure 1. Active thioredoxin inhibitors selected for scale-up and SAR studies.

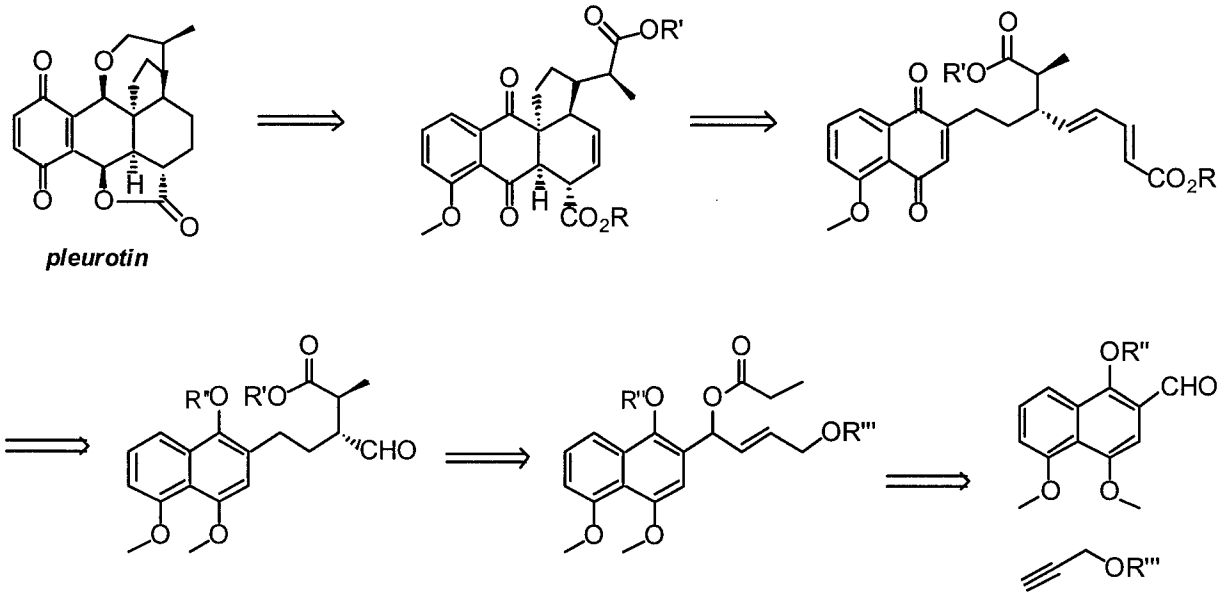


(1) We have successfully scaled up the syntheses of palmarumycin CP_1 and SR-7 (Figure 1) according to the synthetic plan outlined in the last progress report. *In vivo* biological screening of palmarumycin CP_1 has been completed, and SR-7 is scheduled for mouse tumor model efficacy testing in the near future.

(2) We have made considerable progress toward the total synthesis of pleurotin. Also known as NSC401005, this natural product is the most potent Thioredoxin-1/Thioredoxin reductase (Trx-1/TrxR) inhibitor known to date. The IC_{50} of NSC401005 against Trx-1/TrxR was determined as 0.17 μM; however, the average IC_{50} of this compound for growth inhibition in the NCI 60 tumor cell line panel was only 21.5 μM. A total synthesis will

provide us with the foundation for SAR studies of segments and partial structures and the development of more effective analogues. Our synthetic strategy is outlined in Scheme 1. An intramolecular Diels-Alder reaction represents the key step for the formation of the core structure of pleurotin.

Scheme 1. Synthetic strategy for planned pleurotin total synthesis.



Along these lines, our work has progressed well, and we have reached the late stage intermediate **10** (Scheme 2). Most significantly, we have demonstrated that the intramolecular Diels-Alder reaction of **6** is feasible and leads either to the β,γ-unsaturated lactone **7** or the α,β-unsaturated lactone **8**, depending on the amount of sodium borohydride used for the carbonyl reduction step. Lactone **8** was further converted to the saturated pentacycle **10,** which has five of the six rings and six of the eight stereocenters of the target natural product in the correct orientation. Work is now progressing toward adding the α-branched ester side chain and completing the total synthesis.

In addition, during the past year we have become interested in a novel, alkyl 2-imidazolyl disulfide, thioredoxin inhibitor identified by Drs. G. Powis and D. L. Kirkpatrick that has entered Phase I clinical trials in the US. Compounds of this class have been shown to have *in vitro* inhibitory activity against thioredoxin, cytotoxicity to cancer cells *in vitro*, and antitumor activity *in vivo* in animal models. We believe this class of compounds should provide important new information about how an inhibitor of thioredoxin should perform in animals and this has obvious implications to our current project. 1- Methylpropyl-2-imidazoyl disulfide (MID) (Figure 2) is undergoing development as an antineoplastic agent and is currently in Phase I clinical trials. Thus, in conjunction with Drs. Powis and Kirkpatrick and L. Block of Duquesne University, we have developed analytical methods that will permit some modest pharmacodynamic and possibly pharmacokinetic studies to be done with the novel thioredoxin inhibitor. Two peer-reviewed publications [2,3] resulted from this work, which was partially support by this project.

Scheme 2. Summary of our synthetic progress.

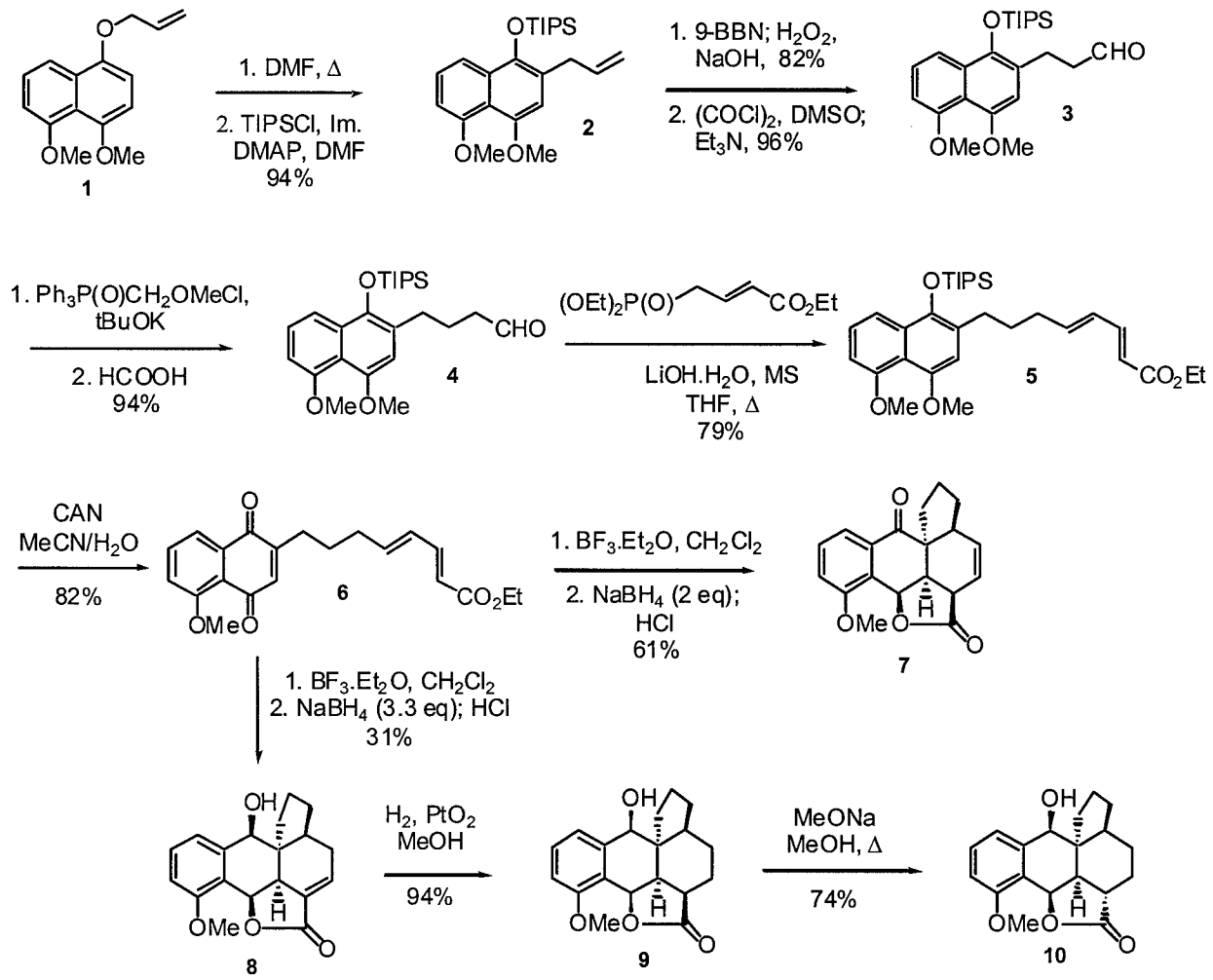
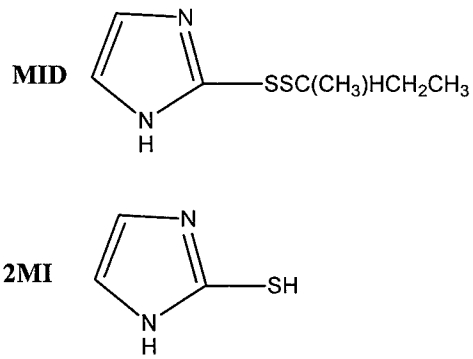


Figure 2. Chemical structures of 1-methylpropyl-2-imidazolyl disulfide (MID) and its putative metabolite 2-mercaptoimidazole (2MI).



Specifically, we developed high-pressure liquid chromatographic (HPLC) methods that allowed us to determine purity, degradation products, metabolite formation in blood and other bodily fluid. The reverse phase HPLC method for MID was linear (r^2> 0.9999), sensitive (LOD=20 ng/ml), reproducible (RSD=0.2%) and accurate (error <1%), MID and it potential metabolite, 2-MI, were both retained on the normal phase HPLC. We also examined the physiochemical characterization of several compounds in this series as an aid to their subsequent formulation and development. Lipophilicity, ionization, and solubility of a number of alkyl 2-imidazolyl

disulfides were studied. Based on the additivity of lipophilicity and ionization properties, the contribution of the unsymmetrical disulfide fragment to lipophilicity and ionization was elucidated. The unsymmetrical disulfide fragment contributed a Rekker's hydrophobic constant of 0.761 to the lipophilicity of these compounds and an approximated Hammett constant (σ) of 0.30 to their ionization. The applicability of the general solubility equation (GSE) proposed by Jain and Yalkowsky in predicting the aqueous solubility of these analogs was evaluated. The GSE correctly ranked the aqueous solubilities of these compounds and estimated their log molar solubilities with an average absolute error of 0.35.

Technical Objective #2. Evaluate the biochemical and cellular activity of library compounds.

With this funding, we have been able to generate sufficient human thioredoxin, thioredoxin reductase, and Cdc25 to allow us to perform complete *in vitro* evaluations on the above-mentioned compounds.

Cdc25. The preparation of plasmid DNA and GST-fusion protein has previously been described [4]. The activity of the GST-fusion $Cdc25B_2$ was measured as previously described [4] in a 96-well microtiter plate using the substrate *o*-methyl fluorescein phosphate (OMFP) (Molecular Probes, Inc., Eugene, OR), which is readily metabolized to the fluorescent *o*-methyl fluorescein. All samples were prepared with a Biomek 2000 automated workstation and the OMFP concentration approximating the K_m for $Cdc25B_2$: 40 μM. All analogs were resuspended in DMSO and all reactions including controls were performed at a final concentration of 7% DMSO. The final incubation mixture (150 μl) was optimized for enzyme activity and comprised 30 mM Tris (pH 8.5), 75 mM NaCl, 1 mM EDTA, 0.033% bovine serum albumin, and 1 mM DTT. Reactions were initiated by adding 1 μg of Cdc25 phosphatase and had 30 μM as the initial concentration tested. Fluorescence emission from the product was measured over a 20-60 min reaction period at ambient temperature with a multiwell plate reader (PerSeptive Biosystems Cytofluor II; Framingham, MA; excitation filter, 485/20; emission filter, 530/30). For all enzymes the reaction was linear over the time used in the experiments and was directly proportional to both the enzyme and substrate concentration. We found none of the compounds caused even a 25% inhibition of enzyme activity.

Thioredoxin and thioredoxin reductase. Thioredoxin reductase was purified from human placenta as previously described [5] and recombinant human Trx-1 was prepared as previously described [6]. TrxR and Trx-1/TrxR activities were measured spectro-photometrically using previously published microtiter plate colorimetric assays, based on the increase in absorbance at 405 nm, which occurs as dithionitrobenzoic acid (DTNB) is reduced by the enzyme-mediated transfer of reducing equivalents from NADPH [6]. Trx-1/TrxR-dependent insulin reducing activity was measured in an incubation with a final volume of 60 μL containing 100 mM HEPES buffer, pH 7.2, 5 mM EDTA (HE buffer), 1 mM NADPH, 1.0 μM TrxR, 0.8 μM Trx-1 and 2.5 mg/mL bovine insulin. Incubations were for 30 min at 37 °C in flat-bottom 96-well microtiter plates. The reaction was stopped by the addition of 100 μL of 6 M guanidine-HCl, 50 mM Tris, pH 8.0, and 10 mM DTNB, and the absorbance measured to 405 nm. TrxR activity was measured in a final incubation volume of 60 μL containing HE buffer, 10 mM DTNB, 1.0 μM TrxR and 1 mM NADPH. Compounds were diluted in HE buffer and added to the wells as 20 μL aliquots, and TrxR (20 μL HE buffer) was then added. Analogs were tested at 0.1 to 50 μM concentration. To start the reaction NADPH and DTNB were added as a 20 μL aliquot in HE buffer and the plate was moved to the plate reader that had been preheated to 37 °C. The optical density at 405 nm was measured every 10 s and initial reaction rates were measured.

Table 1. IC_{50} values [μM] for TrxR, Trx-1/TrxR and cell growth inhibition.[a]

Entry	Compound	TrxR	Trx-1/TrxR	MDA-MB-231	MCF-7
1	**palmarumycin CP_1**	12.0	0.35	2.4	1
2	**JK-2**	nd[b]	8.0	2.1	1.3
3	**JK-3**	nd	2.1	6.4	3.8
4	**JK-4**	nd	12.2	23	4.6
5	**JK-5**	nd	44.0	3.4	1.3
6	**JK-6**	nd	>50	8.2	4.6
7	**JK-7**	nd	13.5	2.9	2.8
8	**diepoxin σ**	13.5	4.5	2	1.5
9	**SR-1**	nd	>50	7.5	7.9
10	**SR-2**	nd	>50	2.9	1.3
11	**SR-3**	nd	>50	13.6	13.4
12	**SR-4**	nd	>50	9.2	>30
13	**SR-5**	nd	>50	2.7	2.3
14	**SR-6**	nd	>50	4.6	3.9
15	**SR-7**	nd	>50	2.5	1.1
16	**SR-9**	nd	>50	2	4.6
17	**SR-10**	>50	23.2	2	2
18	**SR-11**	>50	41.8	2.8	2
19	**SR-12**	>50	>50	1.4	1.5
20	**SR-13**	>50	>50	7.3	8
21	**SR-14**	>50	23.2	2.7	2
22	**TH-39**	>50	>50	2.2	0.7
23	**TH-40**	>50	4.8	8.2	7.8
24	**TH-44**	>50	13.4	4.7	4.3
25	**TH-48**	>50	>50	9.3	>10
26	**TH-49**	>50	>50	8	>10
27	**TH-62**	20.1	10.2	7.8	5.7
28	**TH-63**	>50	>50	>10	>10
29	**TH-64**	>50	>50	>10	>10
30	**TH-65**	>50	>50	4.9	5
31	**TH-66**	>50	42.4	5.2	5.5
32	**TH-126**	nd	>50	3.6	2
33	**TH-139**	nd	>50	5.3	4.3
34	**TH-140**	nd	>50	4.5	1.9
35	**TH-169**	8.8	3.4	4.3	4.2
36	**TH-223**	>50	40.2	5	4.4

[a] **IC_{50} values were calculated from 5 concentrations (0.1 to 50 μM) for both the Trx-1/TrxR and TrxR assays. nd = not determined.**

Table 1 summarizes Trx-1/TrxR assay data. The most active compounds inhibited Trx-1/TrxR with IC_{50} values from 0.35 to low micromolar. In particular, palmarumycin CP_1 rivaled the most active known inhibitor of the thioredoxin system, pleurotin, in activity (entry 1). Palmarumycin CP_1 also demonstrated considerable (>30 fold) selectivity for Trx-1 over TrxR. Alkylation at the phenol as shown in the SR-series of analogs mostly abolished activity, with the exception of **SR-10**, a 3-furylmethyl derivative (entry 17), and **SR-14**, an allylated phenol (entry 21) which were nonetheless >50 fold less active. For the most part, this trend is continued in the TH-series, but several derivatives show more significant affinity to the thioredoxin – thioredoxin reductase system. Specifically, **TH-40** (entry 23), **TH-44** (entry 24), and **TH-62** (entry 27) have IC_{50} values from 4.8 to 13.4 μM. The former two are closely related to **SR-14**, but the activity of **TH-62** is unexpected given the lack of activity of the closely related **TH-63-66**. The beneficial effects of the free phenol group in the palmarumycin pharmacophore for Trx-1/TrxR inhibition are most strikingly demonstrated in the comparison of **TH-169** and **TH-223**. Only the free phenol **TH-169** maintains significant activity (entry 35) while the methyl ether **TH-223**

is practically inactive. The comparison between palmarumycin CP_1 and **TH-169** also demonstrates the contribution to activity by the naphthalenediol ketal; a replacement with the 1,3-dioxolane group decreases activity ~10 fold and, most significantly, reduces the Trx-1 selectivity from >30 to ~2 fold. The presence of the conjugated enone is not crucial, as comparisons in the JK-series demonstrate. The diepoxyketones **JK-7** and, in particular, diepoxin σ, maintain respectable levels of inhibition of Trx-1/TrxR (entries 7 and 8), even though successive replacements of the double bonds in **JK-4**, **JK-5**, and **JK-6** with epoxide rings leads to noticeable decreases in activity vs. the parent dienone **JK-3**. These results are described in more detail in our published manuscript [12]. We are now in the process of synthesizing analogs of pleurotin as second generation inhibitors.

Antiproliferative activity. Antiproliferative activity was examined with estrogen receptor positive, p53-replete, MCF-7 and estrogen receptor negative, p53-deficient, MDA-MB-231 human breast cancer cells using a previously described colorimetric assay [7,8]. Cells were seeded at 4,000 cells/well in 96 well microtiter plates and allowed to attach overnight. Cells were treated with compounds (0.1 to 30 μM) or 0.1% DMSO (vehicle) for 72 h, after which the medium was replaced with serum-free medium containing 0.1% of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. Plates were incubated for 3 h in the dark and the total cell number was calculated spectrophotometrically at 540 nm. Table 1 indicates that a substantial majority of the compounds evaluated with either MDA-MB 231 or MCF-7 cells were quite active as antiproliferative agents. Generally, MCF-7 cells were slightly more sensitive than the p53-deficient MDA-MB-231 cells, although **SR-4**, **SR-9**, **TH-48** and **TH-49** are notable exceptions. We were, however, unable to make any correlation between *in vitro* inhibition of thioredoxin or thioredoxin reductase and growth inhibition suggesting growth may be the result of other biochemical effects. Thus, these are promising cytotoxic compounds whose mechanism of action for growth inhibition remains to be established. We have some evidence that some of these compounds caused growth inhibition because of apoptotic processes.

We have evaluated the cell cycle inhibition caused by SR-7. Using our previously described synchronous tsFT210 mammary carcinoma cells [8], we probed the effects of SR-7 on both G2/M and G1 transition [9]. When incubated at the permissive temperature of 32.0°C, tsFT210 cells had a normal cell cycle distribution [9]; when incubated at the non-permissive temperature of 39.4°C for 17 h, cells arrested at G2/M phase (4C), due to Cdk1 inactivation. When G2/M arrested cells were cultured at the permissive temperature for 6 h with DMSO vehicle alone, we saw clear evidence of entry into G1. To determine the effect of SR-7 on G2/M cell cycle transition, we treated cells with 2.5 to 10 μM SR-7 for 6 h after releasing cells at 32.0°C. SR-7 caused a concentration-dependent arrest in the G2/M phase, with obvious blockage even with 2.5 μM SR-7. Because of this remarkable antiproliferative activity, we have decided to take this compound into further preclinical studies.

We also examined G1 transition in tsFT210 cells after SR-7 treatment. We again arrested tsFT210 cells at G2/M by shifting to the non-permissive temperature and then released cells into G1 phase by returning to the permissive temperature. In these experiments, however, we added DMSO vehicle or SR-7 6 h after G2/M phase release. Cells that were treated with the DMSO vehicle passed through G1 phase as expected and produced the predicted broad S phase peak between diploid (2C) and tetraploid (4C) states, while cells exposed continuously to 50 μM roscovitine were blocked and did not pass through G1. Cells treated with 5 or 10 μM SR-7 were not delayed at G1. Thus, we now have in place a system to evaluate cell cycle block.

Technical Objective #3. Evaluate the antitumor activity of prioritized compounds.

We have begun studies that will allow us to analyze the antitumor activity of any other potent inhibitor of thioredoxin. It is critical that we have the appropriate formulation as well as dosage schedule for each compound. The studies to determine the optimal solubility and formulation of SR-7 are currently being conducted and no results are currently available. As mentioned above, however, we have completed our characterization of another promising thioredoxin inhibitor, which became available to us during the course of this project. This novel compound, MID, was identified as a potent cytotoxic agent toward human tumor cells in culture and has *in vivo* antitumor activity in animals. Thus, we initiated analytical studies described above. Our experience with this compound can now be transferred to SR-7 and any other compound that are prioritized

by our group. We have performed initial studies to determine the maximal tolerated dose of palmarumycin in mice and have seen no obvious toxicity with up to 400 mg/kg ip (single injection). As formulated, however, the compound was in suspension. Therefore, we will need to address the formulation of this compound before we proceed.

Key Research Accomplishments of the Project.

Task 1. Specific Aim #1. Synthesize libraries of small molecule compounds designed on the spiroketal naphthodecalin structure.

- Synthesized an initial library of approximately 50 compounds in 5-20 mg quantities
- Evaluated the purity and confirm chemical structure of library members.
- Scaled up synthesis to permit extensive analysis of prioritized compounds.
- Approached a second-generation library based on the pleurotin structure; made core structures for the total synthesis of pleurotin.

Task 2. Specific Aim #2. Evaluate the biochemical and cellular activity of library compounds.

- Generated sufficient thioredoxin, thioredoxin reductase, and Cdc25 for *in vitro* assays.
- Evaluated the inhibitory activity of the initial library against thioredoxin and thioredoxin reductase *in vitro*.
- Determined the growth inhibitory activity of initial library members against MDA-MB-231 and MCF-7 cells.
- Established cell cycle assay with tsFT210 cells and spiroketals.
- Examine antimitotic actions and antitubulin activity of prioritized compounds.
- Determined the cell cycle actions of library members against MDA-MB-231 and MCF-7 cells.
- Examine the structure-activity relationship (SAR) of initial library members and prioritized compounds for further studies and resynthesis.

Task 3. Specific Aim #3. Evaluate the antitumor activity of prioritized compounds.

- Developed analytical methodology to detect a novel inhibitor of thioredoxin in plasma and tissues.
- At assist in the development of methods that can be used in human clinical trials and animal antitumor studies.
- Determined the solubility, ionization and partitioning behavior of a novel inhibitor of thioredoxin, MID.

Reportable Outcomes

Bibliography of Publications:

Wipf, P., Hopkins, T.D., Jung, J.-K., Rodriguez, S., Birmingham, A., Southwick, E.C., Lazo, J.S. and Powis, G. New inhibitors of thioredoxin-thioredoxin reductase system based on a naphthoquinone spiroketal natural product lead. Bioorg. Med. Chem. Ltr. 11:2637-2641, 2001.

Hashash, A., Kirkpatrick, D.L., Egorin, M.J., Block, L.H. and Lazo, J.S. Nornal-phase and stability –indicating reversed-phase high-performance liquid chromatographic methods for the determination of the novel antitumor agent: 1-methylpropoyl-2-imidazolyldisulfide. J. Chromatog. B. 768:239-246, 2002.

Hashash, A., Kirkpatrick, D.L., Lazo, J.S. and Block, L.H. Solubility, ionization and partitioning behavior of unsymmetrical disulfide compounds: Alkyl 2-imidazolyl disulfides. J. Pharmaceut. Sci. 91:1686-1692, 2002.

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Personnel Supported from the Research Effort:

Name	Degree(s)	[redacted]	Role on Project (e.g., P.I., Res. Assoc.)	[redacted]	Annual % Effort
John S. Lazo	Ph.D.	[redacted]	Principal Investigator	[redacted]	5
Peter J. Wipf	Ph.D.	[redacted]	Co-Investigator	[redacted]	5
Iliya M. Lefterov	M.D., Ph.D.	[redacted]	Research Associate	[redacted]	50
Ahmad Hashash	B.S.	[redacted]	GSR	[redacted]	100

Conclusions

We have developed a general method for the synthesis of multiple napthoquinone spiroketals and have successful scaled up the synthesis for two prioritized compounds. We have demonstrated several novel cytotoxic agents that block human breast cancer cells in G_2/M. We have developed methods to perform pharmacokinetic studies in mice using a known inhibitor of thioredoxin. Initial studies indicate several potent *in vitro* inhibitors of thioredoxin may be useful candidates for future testing in animal models so we are examining these compounds in greater detail. We are being to synthesize second-generation thioredoxin inhibitors based on the natural product pleurotin.

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